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In situ eutrophication stimulates dinitrogen fixation, denitrification, and productivity in Red Sea coral reefs

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ABSTRACT: Eutrophication (i.e. the increase of [in-]organic nutrients) may affect the functioning of coral reefs, but knowledge about the effects on nitrogen (N) cycling and its relationship to productivity within benthic reef communities is scarce. Thus, we investigated how *in situ* manipulated eutrophication impacted productivity along with 2 counteracting N-cycling pathways (dinitrogen [N_2]-fixation, denitrification), using a combined acetylene assay. We hypothesised that N_2-fixation would decrease and denitrification increase in response to eutrophication. N fluxes and productivity (measured as dark and light oxygen fluxes assessed in incubation experiments) were determined for 3 dominant coral reef functional groups (reef sediments, turf algae, and the scleractinian coral *Pocillopora verrucosa*) after 8 wk of *in situ* nutrient enrichment in the central Red Sea. Using slow-release fertiliser, we increased the dissolved inorganic N concentration by up to 7-fold compared to ambient concentrations. Experimental nutrient enrichment stimulated both N_2-fixation and denitrification across all functional groups 2- to 7-fold and 2- to 4-fold, respectively. Productivity doubled in reef sediments and remained stable for turf algae and *P. verrucosa*. Our data therefore suggest that (1) turf algae are major N_2-fixers in coral reefs, while denitrification is widespread among all investigated groups; (2) surprisingly, and contrary to our hypothesis, both N_2-fixation and denitrification are involved in the response to moderate N eutrophication, and (3) stimulated N_2-fixation and denitrification are not directly influenced by productivity. Our findings underline the importance and ubiquity of microbial N cycling in (Red Sea) coral reefs along with its sensitivity to eutrophication.

KEY WORDS: Nitrogen cycle · Climate change · Pollution · Red Sea · Acetylene reduction assay · Acetylene inhibition assay

1. INTRODUCTION

Coastal zones have always attracted humans for various reasons (e.g. resources, transportation and logistics, recreational activities), and migration to coastal cities and areas is increasing (e.g. Neumann et al. 2015). Eutrophication is one of many stressors that intensifies congruently to growing coastal populations (Burke et al. 2011). Coastal nutrient point-sources, i.e. excessive nutrient loads from (un-)controlled sewage dumping or agricultural fertiliser runoff, play a major role in reshaping nearshore eco-

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systems such as coral reefs (e.g. Lapointe 1997, McManus & Polsenberg 2004). Coral reefs, however, are usually adapted to low-nutrient (i.e. oligotrophic) environments, but paradoxically belong to the most diverse and productive ecosystems on earth (Odum & Odum 1955) with nitrogen (N) acting as an important factor limiting productivity (reviewed in Lesser et al. 2007).

Determining the effects of excessive nutrient availability on tropical coral reefs has been part of numerous studies over the last decades, displaying beneficial as well as negative effects. It has been demonstrated that an increase in inorganic matter can shift roles of heterotrophy and autotrophy in coral energetics (Anthony & Fabricius 2000), and that high loads of particulate organic matter do not always negatively impact the physiology of corals (Anthony 2006). Furthermore, enhanced heterotrophic feeding on zooplankton can facilitate coral tissue growth and calcification rates (Ferrier-Pagès et al. 2003), whereas coral starvation can lead to lower photosynthetic activity, congruently with lower lipid and protein concentrations (Borell et al. 2008). Investigations of long-term nutrient enrichment effects revealed threats to coral reefs on multiple levels, ranging from a higher susceptibility of corals to bleaching and mortality (Wiedenmann et al. 2013, Vega Thurber et al. 2014) to negative effects on coral growth and calcification rates (Ferrier-Pagès et al. 2000, Koop et al. 2001) or reproductive success (Harrison & Ward 2001, Loya et al. 2004). When nutrient availability increases over extended periods, phase shifts from coral-dominated reefs to (macro-)algae-dominated states of a reef are likely to occur (e.g. Graham et al. 2015). Moreover, nutrient enrichment together with other anthropogenic stressors that have been shown to alter N-cycling processes, such as ocean warming (Hughes et al. 2018) or ocean acidification (Rädecker et al. 2014, Luo et al. 2019), may result in synergistic effects that ultimately decrease reef resilience and eventually lead to reef degradation or reef losses (e.g. Graham et al. 2015).

For a better understanding of ecosystem functioning in general, and the effects of elevated nutrients on ecosystem functioning in particular, studying N-cycling in coral reefs is of paramount interest. The import of de novo bioavailable N to the system is partly performed by diazotrophs, i.e. microbes capable of fixing atmospheric dinitrogen (N_2) into bioavailable ammonium (NH_4^+). This N_2-fixation (N_2-fix) is crucial for coral reef ecosystems to maintain the N supply and satisfy N demands (e.g. Lesser et al. 2007, Benavides et al. 2016). N_2-fix rates in coral reefs can fluctuate seasonally and in response to variation in environmental conditions (Cardini et al. 2016). At the same time, microbial denitrification (DENI) removes bioavailable N from the ecosystem as it facilitates the reduction of nitrate (NO_3^-) to N_2 and can thus be described as a counteracting pathway to N_2-fix (Vitousek et al. 1997, Silvennoinen et al. 2008). However, knowledge about DENI in coral reef environments is scarce. Hypothetically, DENI is vital to coral reef ecosystem functioning, especially under eutrophic conditions, as this process removes excess N from the reef system (Koop et al. 2001, Rädecker et al. 2015).

N-cycling activity in coral reefs depends on environmental nutrient availability (Koop et al. 2001, Cardini et al. 2016) that can naturally be shaped by upwelling (Radice et al. 2019), terrestrial runoff after rainfalls (den Haan et al. 2016), or anthropogenic sources (e.g. Loya et al. 2004, Peña-García et al. 2014). As such, N-cycling in coral reef environments has the potential to exacerbate or attenuate eutrophication events. However, little is known about N-cycling in coral reefs under elevated nutrient availability. We hypothesised significant responses of N_2-fix and DENI rates to eutrophication (Joye & Paerl 1993). In case of N_2-fix, we expected decreasing activity in eutrophic environments, as hypothesised previously (Cardini et al. 2014). Koop et al. (2001) were able to demonstrate this in coral reef-associated sediments of the Great Barrier Reef. This phenomenon was attributed to the idea that bioavailable N, e.g. in the form of NH_4^+ or NO_3^-, offers a more cost-efficient, alternative source of N to an organism (Holl & Montoya 2005, Knapp 2012). Furthermore, the presence of fixed N potentially inhibits the enzyme (nitrogenase) activity responsible for N_2-fix (Fay 1992, Knapp 2012). For DENI, we expected an increase with increasing nutrient availability in accordance with other studies (Joye & Paerl 1993, Rädecker et al. 2015) to subsequently sustain favourable N-limitation (Wiedenmann et al. 2013). Overall, we anticipated a dynamic interplay of N_2-fix and DENI which counterbalances changes in environmental dissolved inorganic nitrogen (DIN) availability.

Here, we aimed to assess the effects of elevated nutrient availability on N-cycling in a coral reef. We used an 8 wk *in situ* nutrient manipulation experiment in natural reef communities in the oligotrophic central Red Sea to investigate the effects of eutrophication on several metabolic processes (N_2-fix, DENI, respiration, and photosynthesis) in a comparative framework with 3 major functional groups: scleractinian coral, filamentous turf algae, and carbonate reef sediments.

2. MATERIALS AND METHODS

2.1. Experimental design

The *in situ* manipulation experiment was conducted from late January until late March 2018 in a semi-exposed area of the Abu Shosha reef in the Jeddah Region (22° 18′ 15″ N, 39° 02′ 56″ E) on the west coast of Saudi Arabia in the central Red Sea. In total, 8 distinct (i.e. >5 m apart from each other) natural reef communities at a water depth of 5–6 m were chosen; 4 slow-release fertiliser tubes (Osmocote Plus [15-9-12]) were attached with pins around each reef community (Fig. 1). Osmocote Plus fertiliser continuously supplied various macronutrients — 15% total N, 9% available phosphorus (P) in the form of phosphate (PO_4^{3-}), 12% soluble potash (a detailed list of released micronutrients can be found in Table S1 in the Supplement at www.int-res.com/articles/suppl/m645p055_supp.pdf) — from the first day of fertilisation under local temperature regimes (Adams et al. 2013), and has been successfully utilised in previous eutrophication studies (Falkenberg et al. 2013, Stuhldreier et al. 2015). As nutrients tend to leach out during the first weeks (Adams et al. 2013) and to reduce resulting bias, fertiliser pins were renewed every 2 wk to ensure continuous nutrient inputs. To account for dilution effects with surrounding waters, water samples from different distances to the fertiliser were taken every second week to quantify nutrient concentrations. Specimens of target organisms and substrates were taken from close radius (max. 25 cm) around the fertiliser. The eutrophication phase lasted for 8 wk, and specimens of the fertilised communities (hereafter 'eutrophied communities') were taken at the end of the manipulation phase. Specimens from the surrounding, non-fertilised reef communities (hereafter 'control communities') were taken for comparative analysis at the same time.

2.2. Collection and maintenance

We tested 3 of the most dominant biotic and abiotic functional groups of benthic reef communities of the Central Red Sea (carbonaceous reef sediments, filamentous turf algae, and the scleractinian coral *Pocillopora verrucosa*). These 3 groups contribute to more than 70% of the benthic community composition of the sampled reef (Roth et al. 2018). Sediments were collected using a Petri dish (material: polystyrene; diameter: 5.5 cm; height: 1.4 cm) that was pushed carefully into the sediment. Sediments were then fixed to the dish from underneath so that upper sediment 'cores' with a max. sediment depth of 14 mm were sampled. Turf algae were defined as dead coral

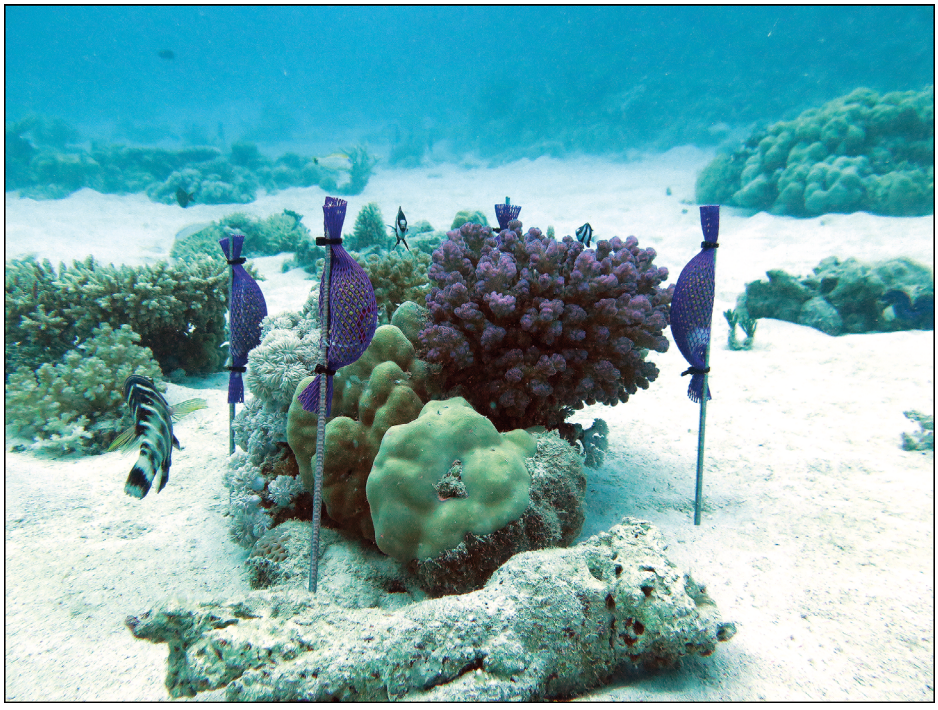

Fig. 1. Manipulated *in situ* community on Abu Shosha reef, central Red Sea. Four pins with attached fertiliser bags. Photo: Florian Roth; reproduced from Karcher et al. (2020), under the CC-BY 4.0 License

fragments of approx. 10 cm length, overgrown with dense and flat (<2 cm in height) assemblages of filamentous algae of different species, including small individuals of macroalgae and cyanobacteria. Examples from Northern Red Sea studies have shown that turf algae account for the highest fraction (up to 90%) of benthic algal cover (Haas et al. 2010). Fragments were collected with hammer and chisel. *P. verrucosa* fragments were approx. 10 cm long and were collected with the same tools from different coral colonies to ensure genetic variability. All fragments and Petri dishes containing reef sediment samples were immediately transferred to recirculation aquaria on the boat after sampling (n = 4 from eutrophied communities into aquaria each filled with 10 l of 5 µM NO_3 enriched seawater; n = 5 from control communities into ambient seawater; NO_3^- enrichment consisted of previously prepared sodium nitrate [$NaNO_3$] stock solution, prepared with MilliQ water and $NaNO_3$, ≥99.0%, Sigma-Aldrich) and kept at ambient water temperature and light conditions until the experimental incubations started within 3 h after sampling.

2.3. Environmental parameters

Key environmental parameters were assessed every second week throughout the total manipulation period of 8 wk. Details for analytical approaches are described in Roth et al. (2018). Briefly, seawater temperature was measured continuously with data loggers (Onset HOBO Water Temperature Pro v2 Data Logger, U22-001; accuracy: ±0.021°C). Seawater samples for assessing concentrations of NO_3^-, nitrite (NO_2^-), and phosphate (PO_4^{3-}) were taken from various distances from fertiliser pins (i.e. directly at the fertiliser pins, 25 cm from inside the communities, >200 cm outside the communities serving as controls). Water samples were filtered immediately on the boat (Isopore™ GTTP membrane filters, 0.2 µm) and the filtrate was stored at 4°C in the dark until frozen at –50°C in the lab within 3 h after collection. Nutrient concentrations were determined using a continuous flow analyser (AA3, HR, SEAL), following colourimetric standard methods (Grasshoff et al. 1999). Limits of quantification (LOQ) for NO_3^-, NO_2^-, and PO_4^{3-} were 0.084, 0.011, and 0.043 µmol l^{-1}, respectively. From these seawater samples, 5 ml subsamples were taken for NH_4^+ determination using the ortho-phthaldialdehyde (OPA) method (Holmes et al. 1999, Taylor et al. 2007). Samples were filtered into separate acid-washed centrifuge tubes, and 1.2 ml OPA solution was added. Samples were then stored for >4 h in the dark. NH_4^+ was determined fluorometrically within 8 h (Trilogy® Laboratory Fluorometer, Turner Designs). The LOQ for NH_4^+ was 0.094 µmol l^{-1}. NO_3^-, NO_2^-, and NH_4^+ were measured in combination (as DIN) and are presented as mean ± SE µM N.

2.4. Primary production, N_2-fix, and DENI measurements

Incubations were conducted *ex situ* and <3 h after sample collection. For O_2 flux measurements, incubation chambers (1 l volume) were filled exclusively with ambient seawater collected the same day (n = 5, with specimens from control communities), and 4 incubation chambers were filled with seawater and amended with 5 µM NO_3^- to provide and keep eutrophic conditions (n = 4, with specimens from eutrophic communities). Additionally, 2 chambers without specimens (one filled with seawater, one filled with 5 µM NO_3-enriched seawater) served as controls to correct for planktonic background metabolism. All chambers were sealed gastight and without any air enclosure. During incubations, the incubation chambers were placed in a tempered water bath and constantly stirred (500 rpm) to ensure stable measurement conditions (27°C). A 2 h light (photon flux of ~200 µM quanta m^{-2} s^{-1}) incubation was followed by a 2 h dark incubation with fresh ambient and nutrient-enriched seawater, respectively. O_2 levels were measured immediately before starting the respective incubations and after 2 h using a WTW Multi 3430 which was equipped with a WTW DFO 925 oxygen sensor. Measured values from dark and light incubations were used to calculate dark respiration (R_{dark}) and net primary production (P_{net}): O_2 start concentrations were subtracted from end concentrations and results were then normalised to incubation time. In the next step, O_2 fluxes were corrected for the seawater control signal, related to incubation volume, and normalised to the surface area of the organisms/substrates. Surface areas for turf algae fragments and *P. verrucosa* were calculated using cloud-based 3-dimensional (3D) models of samples (Autodesk Remake v19.1.1.2; Gutierrez-Heredia et al. 2016). Surface areas of sediments were mathematically calculated (surface area = π × radius^2) as Petri dishes were used for sampling sediment cores. Subsequently, gross primary production (P_{gross}) rates were calculated according to $P_{gross} = P_{net} - |R_{dark}|$.

N_2-fix and DENI incubations were performed using a combined blockage/reduction acetylene assay (COBRA) with the same specimens 3–4 h after the O_2 flux measurements. The incubations were performed as described previously in El-Khaled et al. (2020). Briefly, all COBRA incubations were conducted in gas-tight 1 l glass chambers, each filled with 800 ml of nutrient-enriched seawater (5 µM NO_3^-) and 200 ml headspace. Nutrient-enriched seawater was used in all treatments, as acetylene inhibits the production of NO_3^- in the nitrification pathway (Oremland & Capone 1988). As NO_3^- serves as a substrate for DENI, the inhibition of nitrification potentially results in an underestimation of DENI rates. To compensate for that, and to provide an incubation environment similar to eutrophic reef communities, NO_3^- was added to the incubation water to preclude substrate limitation (e.g. Joye & Paerl 1993, Devol 2008). Both incubation water and headspace were 10% acetylene-enriched. Acetylene (1) leads to a preferential reduction of acetylene to ethylene (C_2H_4) instead of N_2 to NH_4^+ by the nitrogenase enzyme (Balderston et al. 1976), and (2) inhibits DENI at the nitrous oxide (N_2O) stage leading to an evolution of N_2O (Yoshinari & Knowles 1976). Each chamber contained a single sample (scleractinian coral fragment, turf algae, or reef sediment core). In total, 4 or 5 replicate samples were incubated, and an additional 2 chambers without specimens served as controls to correct for planktonic background metabolism. During the 24 h incubations, chambers were submersed in a tempered water bath and stirred continuously (500 rpm) to ensure stable physical conditions and homogenous environment (27°C, 12 h light:12 h dark cycle, photon flux of ~200 µM quanta m^{-2} s^{-1}). Samples were taken at the beginning (t_0) and after 24 h (t_{24}). Both N_2O (for DENI quantification) and C_2H_4 (for N_2-fix quantification) concentrations were quantified by gas chromatography and helium pulsed discharge detector (Agilent 7890B GC system with HP-Plot/Q column; lower detection limits for both target gases: 0.3 ppm). Gas fluxes were corrected for the seawater control signal and normalised to the surface area of the organisms/substrates. C_2H_4 fluxes were converted into corresponding N_2 fluxes assuming a theoretical molar ratio of C_2H_4:N_2 = 4, which has been used in previous studies in similar environments (e.g. Rix et al. 2015, Cardini et al. 2016).

2.5. Statistical analysis

The data set was analysed using non-parametric permutational multivariate analysis of variance (PERMANOVA) using PRIMER-E v6 software (Clarke & Gorley 2006) with the PERMANOVA+ add-on (Anderson 2001). To test for differences in N_2-fix, DENI, and O_2 fluxes between functional groups and eutrophied and control communities, 2-factorial PERMANOVAs were performed (factors were 'functional group' and 'control/eutrophied reef community'), based on Bray-Curtis similarities of square-root transformed data. Therefore, Type III (partial) sum of squares was used with unrestricted permutation of raw data (999 permutations), and PERMANOVA pairwise tests with parallel Monte Carlo tests were carried out when significant differences occurred. To identify correlations between N_2-fix, DENI, P_{gross}, P_{net}, and R_{dark}, a Spearman-rank-order correlation was performed using SigmaPlot for Windows v12.0.

3. RESULTS

3.1. Environmental parameters

Water temperature increased from 24.8–28.1°C during the *in situ* manipulation period of 8 wk. Background DIN concentrations remained stable throughout the experiment at 0.40 ± 0.03 µM N. When nutrient manipulation was initiated, DIN concentrations increased up to 7-fold (measured directly at the communities) compared to background values (Fig. 2). At the same time, PO_4^{3-} remained stable. For

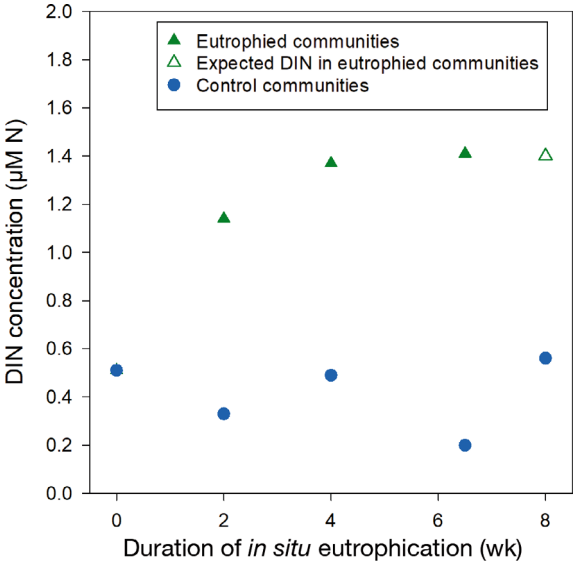

Fig. 2. Dissolved inorganic nitrogen (DIN) concentrations in control and eutrophied communities. Due to technical issues, no DIN concentrations were determined in eutrophied communities after 8 wk. Expected DIN concentrations, based on previous measurements are displayed

a detailed summary of nutrient concentrations at the centre of eutrophied communities, directly at the fertiliser pins, and in surrounding waters, see Table S2.

3.2. Oxygen fluxes

In both eutrophied and control communities (Fig. 3), reef sediments showed lowest P_{gross}, being 3–4 times lower than in turf algae and 5–6 times lower than in *Pocillopora verrucosa*. A significant increase in P_{gross} between control and eutrophied reef communities was only detected for reef sediments (Table S3).

3.3. N_2-fix and DENI

Concentrations for both C_2H_4 and N_2O control incubations over the incubation time of 24 h were stable (Fig. S1). In control communities, turf algae showed the highest N_2-fix activity (13.68 ± 1.42 nmol N_2 cm^{-2} d^{-1}) among the investigated functional groups, with N_2-fix rates 13 times higher than of sediments (Table S4) and 274 times higher than the investigated scleractinian coral (Fig. 4, Table S4). DENI rates did not differ significantly between investigated groups due to high variation, with *P. verrucosa* showing DENI rates 2 times lower than turf algae and 2–3 times lower than reef sediments.

In eutrophied communities, N_2-fix rates of turf algae (27.12 ± 1.57 nmol N_2 cm^{-2} d^{-1}) were 3 times higher than those of sediments (Fig. 4, Table S4) and 2 orders of magnitude higher than those of *P. verrucosa* (Fig. 4, Table S4). For sediments in manipulated reef communities, significantly higher N_2-fix rates were observed compared to control communities (Table S4), being 8-fold higher than without nutrient manipulation. Similar patterns of increasing N_2-fix activity were observed for turf algae and the scleractinian coral of eutrophic communities, though these were not statistically significant. In eutrophied communities, no statistical difference in DENI rates between functional groups was observed. However, compared to untreated specimens, DENI rates in functional groups from eutrophied reef communities increased significantly for turf algae (0.39 ± 0.03; Table S3). DENI rates increased, though not significantly (Table S3), 3-fold in sediments (0.49 ± 0.08) and 5-fold for *P. verrucosa* (0.20 ± 0.06) compared to control communities.

We observed positive correlations between N_2-fix and DENI (Table 1; correlation coefficient $R_s = 0.480$, $p = 0.012$), and between N_2-fix and R_{dark} (Table 1; $R_s = 0.454$, $p = 0.018$), and P_{net} and P_{gross} (Table 1; $R_s = 0.843$, $p < 0.001$). Negative correlations were identified for R_{dark} and P_{net} (Table 1; $R_s = -0.621$, $p < 0.001$), along with R_{dark} and P_{gross} (Table 1; $R_s = -0.891$, $p < 0.001$).

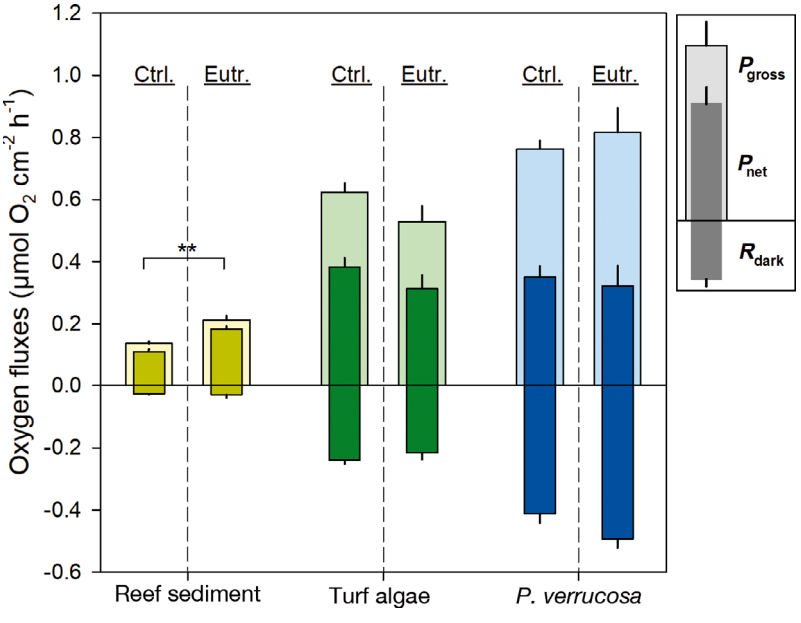

Fig. 3. Dark respiration (R_{dark}), net primary production (P_{net}), and gross primary production (P_{gross}) of 3 functional groups from control (Ctrl.; n = 5) and eutrophied (Eutr.; n = 4) communities. Asterisks indicate significant differences in P_{gross} (**$p < 0.01$). Data are means of replicates ± SE

4. DISCUSSION

Anthropogenically induced nutrient inputs to coral reefs have multi-level impacts (D'Angelo & Wiedenmann 2014, Vega Thurber et al. 2014). This study extends the previous works of Koop et al. (2001) and Capone et al. (1992) by showing that increasing DIN concentrations alter essential biochemical processes such as primary productivity, N_2-fix, and DENI in coral reef communities.

4.1. Environmental parameters

As background DIN concentrations remained stable throughout the experiment (Fig. 2), we are confident that further pulses of nutrient inputs, e.g. through terrestrial runoffs, were

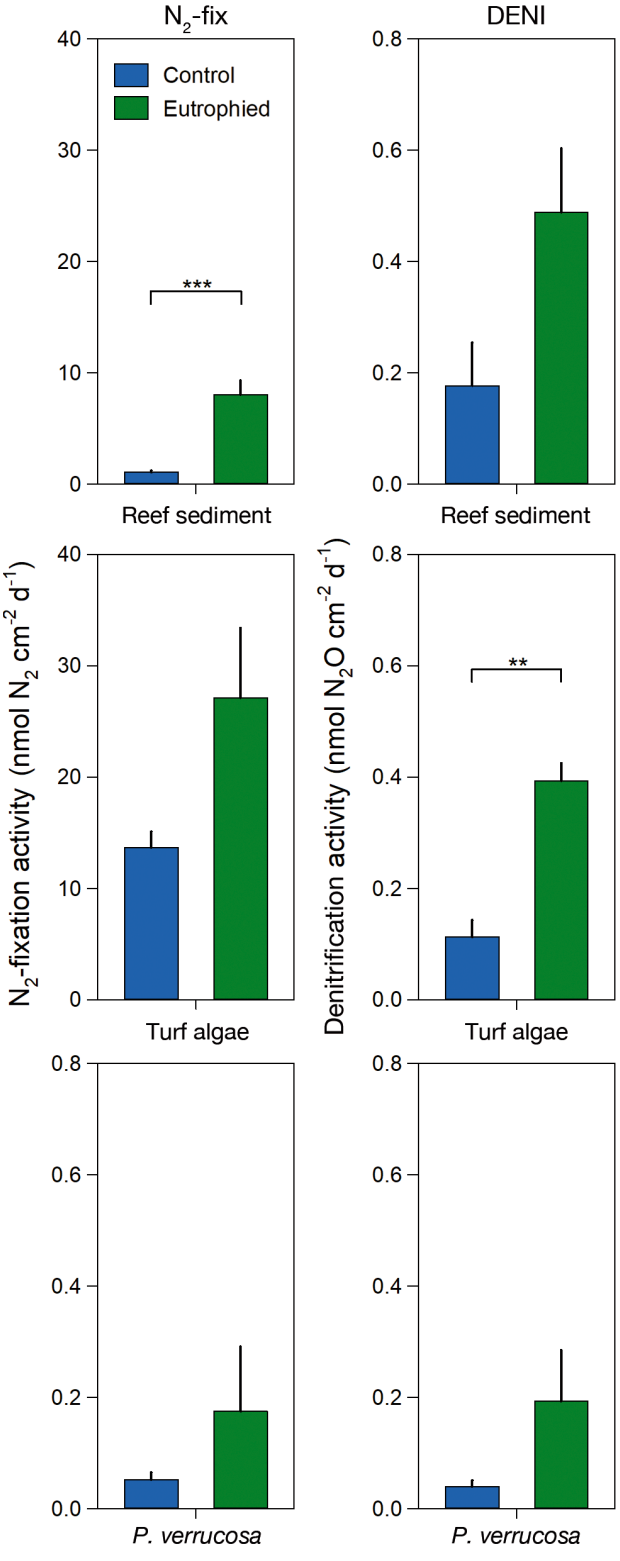

Fig. 4. N_2-fixation (N_2-fix) and denitrification (DENI) activity of investigated functional groups from control (n = 5) and eutrophied (n = 4) communities. Asterisks indicate significant differences (**p < 0.01; ***p < 0.001). Data are means of replicates ± SE. Note different scale for denitrification and N_2-fixation and for reef sediment and turf algae, as they differ from *P. verrucosa*

Table 1. Spearman rank-order correlation coefficients between N_2-fixation, denitrification, dark respiration (R_{dark}), net primary production (P_{net}), and gross primary production (P_{gross}). Pairs of variables with positive correlation coefficients and significant p-values (p < 0.05) tend to increase together; for pairs with a negative correlation coefficient and p < 0.05, one variable tends to decrease while the other increases. *p < 0.05; ***p < 0.001

	Denitrification	R_{dark}	P_{net}	P_{gross}
N_2-fixation	0.480*	0.454*	0.116	−0.311
Denitrification		0.332	−0.221	−0.256
R_{dark}			−0.621***	−0.891***
P_{net}				0.843***

not present. DIN concentrations in experimentally nutrient-enriched communities constantly exceeded concentrations of control communities and were up to 7-fold higher. Compared to other studies, in which manipulative eutrophication was performed (e.g. Koop et al. 2001, Wiedenmann et al. 2013), DIN concentrations in eutrophied communities of the experiments in the present study were about 4–39 times lower and reflect a more realistic ecological scenario in the context of the oligotrophic Red Sea (Churchill et al. 2014, Wafar et al. 2016). We simulated eutrophication in a season when DIN concentrations are usually low (compare to Roth et al. 2018). The manipulation can, thus, be considered as an unnaturally appearing moderate eutrophication event simulating nutrient inputs in the Red Sea from point-sources such as aquaculture (e.g. Loya et al. 2004) or urban wastewater (e.g. Peña-García et al. 2014). Still, eutrophied communities in the present study experienced DIN concentrations that were higher than the 'eutrophication thresholds' of ~1.0 µM suggested by Lapointe (1997), which was also referred to in a previous study in the Red Sea (Jessen et al. 2013), confirming a successful enrichment over the 8 wk manipulation period.

4.2. N_2-fix and DENI under elevated nutrients

Results of the present study showed increasing N_2-fix and DENI in all functional groups in response to higher nutrient availability compared to control communities. High variability caused a lack of statistical significances which could be explained by low replication (n = 4 or n = 5, respectively). Descriptive trends for increasing N_2-fix and DENI were homogenous for all functional groups of eutrophied commu-

nities, and are referred to hereafter when discussed. Potentially, high variability could be counteracted by higher replication. For N_2-fix, these findings contradict our expectations and observations from previous studies that found reduced N_2-fix activities under high DIN availability in coral reef-associated sediments (Koop et al. 2001, Knapp 2012 and references therein). For DENI, this is in line with other studies that have demonstrated increased DENI activities in coral reef-associated sediments under elevated nutrient availability (Capone et al. 1992, Koop et al. 2001).

Theoretically, an energetically more cost-efficient alternative to N_2-fix is the assimilation of N (in the form of NO_3^- and/or NH_4^+), which was provided by performed *in situ* manipulation (Holl & Montoya 2005, Knapp 2012). As a result, we expected a lower N_2-fix activity. However, a stimulating effect on N_2-fix in response to increased DIN in surrounding waters was observed. Generally, the activity of diazotrophs is inhibited by elevated N availability, as shown in mesotrophic or eutrophic lakes (Flett et al. 1980), sediments in bays (Joye & Paerl 1993), seagrass roots in an estuary (Welsh et al. 1996), and in agricultural crops (Vadez et al. 2000). However, the role of nutrients on N_2-fix remains arguable, as there is also continued N_2-fix activity in response to elevated DIN concentrations of up to 30 µM NO_3 (reviewed in Knapp 2012). Even stimulating effects by providing N have been observed (Capone et al. 1990), likely explained by the added form of combined N (chloramphenicol) and the time of day when respective incubations were initiated (afternoon).

The converse response of N_2-fix to elevated N concentrations reported by many studies highlights that N-cycling processes are impacted by multiple environmental factors. For example, in general, both N-cycling processes are performed in anaerobic milieus: elevated O_2 concentrations can result in a depression of nitrogenase activity and subsequently in lower N_2-fix activity (Bebout et al. 1987); likewise, DENI is mediated by anaerobic bacteria (Cornwell et al. 1999 and references therein) and thus also depends (besides other factors) on low O_2 availability (Piña-Ochoa & Álvarez-Cobelas 2006). Therefore, we tested whether O_2 concentrations explained the changes in both N-cycling pathways (Brandes et al. 2007). However, the lack of a correlation between N-cycling pathways and P_{net} and/or P_{gross} excluded O_2 as a potential driver for both N_2-fix and DENI activity. Therefore, we hypothesise that both processes may be spatially or temporally separated from O_2 evolution in reef sediments, turf algae, and *Pocillopora verrucosa* (Tilstra et al. 2019). A temporal separation in hard corals can occur due to heavily varying O_2 concentrations within boundary layers, ranging from super-saturation during daylight to anoxia at night, caused by metabolic processes of the coral host and Symbiodiniaceae (Shashar et al. 1993). These alternating changes from anaerobic to aerobic conditions can even fuel DENI, as nitrification (i.e. the oxidation from NH_4^+ to NO_2^- and NO_3^-) is stimulated by the presence of O_2 (Rysgaard et al. 1994), subsequently resulting in the formation of NO_3^- which serves as a substrate for DENI (Devol 2008). Alternatively, increased N_2-fix and DENI in all groups indicate that the involved N-cycling prokaryotes are capable of performing N_2-fix and DENI in the presence of O_2 (Silvennoinen et al. 2008). From this finding, we conclude that N_2-fix and DENI activities were not directly altered by the presence of O_2.

Besides an oxic–anoxic environment, the availability of organic carbon (C_{org}) can be decisive for diazotrophs and denitrifiers, as it poses as an important energy source (Beauchamp et al. 1989, Lema et al. 2012). C_{org} can be acquired via uptake from the water column (Sorokin 1973) or, in coral holobionts, translocated by C-rich photosynthates from symbionts (Rädecker et al. 2015 and references therein).

We report elevated P_{gross} activity in reef sediments of eutrophied communities compared to the control, suggesting increased C_{org} in reef sediments, which was potentially caused by higher photosynthetic rates of epilithic algae on the sediment as described by Cook et al. (2007). Additionally, the export of C_{org} from neighbouring turf algal assemblages in the form of dissolved organic carbon (DOC) (Haas et al. 2011), with a subsequent DOC uptake by reef sediments (Cárdenas et al. 2015), potentially provides sufficient C_{org} as an energy source leading to increasing N_2-fix activities. Thus, we conclude that even though N of the fertiliser pins was not taken up by reef sediments directly (Karcher et al. 2020), an indirect effect via the interplay with other functional groups led to a stimulation of N_2-fix. This, along with the findings of a related study (Karcher et al. 2020), indicates that the reef sediments and their associated microbial community were not N-limited.

For turf algae, DENI rates measured in eutrophied communities were significantly higher than those from control communities. Similarly, N_2-fix was increased in turf algae from eutrophied communities. Our data reveal no changes in P_{gross}, P_{net}, or R_{dark} in turf algae from eutrophied communities compared to controls; thus, no further (direct) source of energy was provided for N-cycling processes. Turf algae can, however, be highly flexible in acquiring N from

different sources. For example, N_2-fix in turf algae was highest among all measured functional groups; thus, this process contributed considerably to satisfy N demands in control communities (Yamamuro et al. 1995, Rix et al. 2015). Shifts to a preferable uptake of allochthonous N in eutrophied communities (Karcher et al. 2020) were detected, emphasising the turf algae's flexibility and underlining their role as opportunists efficiently taking up environmentally offered N (den Haan et al. 2016). We support the idea of turf algae assemblages being N-limited and benefiting from increased DIN concentrations. In this context, the ability to rapidly take up N (den Haan et al. 2016) as well as assimilate and process N compounds can subsequently result in rapid takeover of bare substrates (Stuhldreier et al. 2015, Roth et al. 2018), which underlines the competitiveness of turf algae under elevated nutrient concentrations (Gorgula & Connell 2004). Thus, an assumed incorporation and processing of N can ultimately enhance microbial growth, as assimilates can be stored or used for metabolic processes (Kopp et al. 2013). We suggest a conterminously increasing abundance of diazotrophs (Muscatine et al. 1989) and likely denitrifiers, which could explain the increased N_2-fix and DENI activities, although microbial communities may vary and respond strongly to environmental changes.

In *P. verrucosa*, we observed increasing N_2-fix and DENI rates in eutrophied communities compared to controls. Stimulated N_2-fix by elevated DIN concentrations contradicts expected patterns. Potentially, longer experiments (36 mo; Vega Thurber et al. 2014) and/or longer experiments mimicking a more severe eutrophication event (12 mo, 36.2 µM NH_4^+; Koop et al. 2001) could lead to suppressed N_2-fix rates in coral holobionts. This underlines that elevated nutrient concentrations do not necessarily negatively impact coral holobionts under certain conditions. Indeed, Atkinson et al. (1995) demonstrated that hard corals can flourish across a wide range of nutrients without showing signs of stress. Moreover, Bongiorni et al. (2003) showed that increased nutrient availability can promote coral growth. Thus, we speculate that nutrient enrichment (i.e. eutrophication) may not always negatively impact scleractinian corals (Lirman & Fong 2007, Sawall et al. 2011). Additionally, it has been demonstrated that *P. verrucosa* harbours a stable, rather inflexible microbiome (Pogoreutz et al. 2017). This ultimately questions whether the N_2-fix pathway is continually facultative as previously assumed (Rädecker et al. 2015) or if it can be considered obligate to some extent under certain conditions — a N_2-fix strategy as has been observed in terrestrial ecosystems (Menge et al. 2009). Moreover, stimulated DENI and N_2-fix activity through increased DIN concentrations suggest that both DENI and N_2-fix may potentially be carried out — at least to a certain degree — by the same microbes, as previously postulated for seagrass-associated bacteria (Patriquin 1978, Bothe et al. 1981). Furthermore, we assume a shift from originally N- (Lapointe 1997, Lesser et al. 2007, Eyre et al. 2008) towards P-limitation (Wiedenmann et al. 2013) and/or other micronutrients (D'Angelo & Wiedenmann 2014, Luo et al. 2019) in *P. verrucosa* from treated communities. This likely occurs when excess inorganic N is available (Wiedenmann et al. 2013). Here, we utilised fertiliser bags for manipulation that also contained P. Although P concentrations were increased at the fertiliser pins directly (Table S2), the role of P in our study remains speculative, as no increase inside the eutrophied communities was observed. This can be explained either by (1) an immediate uptake by benthic and pelagic organisms (Fabricius 2005, Ferrier-Pagès et al. 2016), as P is considered crucial (Cuet et al. 2011) and limiting (e.g. Eyre et al. 2008, Bednarz et al. 2017) for primary productivity; or (2) a discontinuous P supply from the fertiliser due to solubility and quick leaching. By all means, N_2-fix is often limited by micronutrients (e.g. Luo et al. 2019), so that even minor changes in their availability may result in N_2-fix stimulation, as reported for many marine and limnetic systems (Howarth et al. 1988). The assessment of P-enrichment effects on coral reefs by D'Angelo & Wiedenmann (2014) supports this hypothesis, as they described the complex dimension of a rapid P utilisation by N-fixing *Trichodesmium* transforming high P levels into P-depleted conditions.

4.3. Ecological implications

This is the first study to show that microbial N cycling in tropical coral reef communities may not provide effective relief from, and may even exacerbate, anthropogenic eutrophication due to stimulated N_2-fix. We further posit that N_2-fixers are rather inflexible in their response to anthropogenic N inputs (i.e. increasing N inputs to coastal ecosystems, e.g. Lapointe 1997) under certain conditions. Extrapolating these findings in the light of climate change, we suggest that microbial N cycling may contribute to N oversupply and thereby increase the likelihood of phase shifts from coral-dominated to algae-dominated reefs (McManus & Polsenberg 2004).

Released nutrients from the fertiliser pins added to a low baseline (in terms of background nutrient concentrations), even though final DIN concentrations did not exceed naturally occurring DIN fluctuations of the same reef (Roth et al. 2018). Thus, the observed N_2-fix stimulation in the present study conceivably occurred due to an inflexibility to respond to anthropogenically induced DIN changes (i.e. an expected reduction of the energetically costly N_2-fix pathway), even though the capacity to react to natural DIN inconstancies has been demonstrated before (Rix et al. 2015, Cardini et al. 2016). Additionally, we suspect that specific DIN thresholds, which regulate the activity of N_2-fix (and subsequently also of DENI), were not exceeded in the present study.

Moreover, DENI is only one mechanism within the N cycle that can relieve coral reef ecosystems of excessive N. Further processes, such as anaerobic NH_4^+ oxidation (ANAMMOX) transform fixed NH_4^+ into elemental N_2, thereby removing bioavailable N from the system. ANAMMOX occurs in many marine environments (Dalsgaard et al. 2005, Brunner et al. 2013) and has been detected in coral reef-associated sponges (Hoffmann et al. 2009). It also likely appears in coral holobionts (Rädecker et al. 2015), for which the role of ANAMMOX needs to be targeted in future studies.

Synoptically, future research should thus aim to (1) address the effects of severe eutrophication on N_2-fix and DENI in coral reef communities and identify DIN thresholds by which N_2-fix is suppressed; (2) detect and quantify the interaction between N_2-fix and DENI with other N-cycling pathways (i.e. nitrification and ANAMMOX); and (3) identify a coral reef community-wide N_2-fix and DENI budget under ambient and stressed scenarios, precisely because the role of symbiotic and planktonic diazotrophs providing fixed N in bleached corals is still under debate (Bednarz et al. 2017, Meunier et al. 2019).

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